Plant regeneration from an endangered valuable cork oak tree by somatic embryogenesis

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ABSTRACT

Among the several applications of *in vitro* tissue culture techniques, the conservation of plant germplasm is one of the most widely used.

The cork oak is one of the principal tree species in the Western Mediterranean region. Within this area, the Balearic Islands are considered to be a glacial refuge, and therefore a reservoir of genetic resources. A singular tree has been found in the small Minorca Island population. The haplotype of this tree is of Tyrrhenian origin, showing a past link between Minorca and Sardinia. Moreover, this tree do not bear a deletion within an ITS from ribosomic nuclear DNA, which is fairly common in many populations of this species, and indicates that it may be the descendant of a very ancient population. This tree is currently in a precarious condition, and it has not produced acorns in the last years. Hence there is a clear need of vegetative propagation to conserve this genotype. We have previously developed methods to clone adult cork oak trees by somatic embryogenesis, and therefore the aim of the present work was to clone this singular tree. Three branches from the crown were collected in November 2004, and methods previously described were carried out. By February 2005 somatic embryogenesis was obtained from leaves of the tree with percentages of induction ranging from 17 to 54 % depending on the branch, which may show a novel source of variation that requires further study. Spontaneously matured somatic embryos germinated at 46 % in average, and the first somatic seedlings from the Alfavaret's cork oak tree were obtained. Therefore, this study shows one of the most relevant applications of somatic embryogenesis: the plant regeneration of valuable genotypes for the *in situ* and *ex situ* conservation of forest genetic resources.

Keywords: Biotechnology, Plant tissue culture, Micropropagation, Somatic embryogenesis, Forest genetic resources

Introduction

Plant biotechnology is undergoing an impressive development in recent years (Campbell et al., 2003). It provides many different tools for application in genetic improvement programs of forest species (El-Kassaby, 2004). As a consequence, FAO recently encouraged the dissemination of information on the applicability of this topic to developing countries on the basis of current developments (FAO, 2004). Among the different tools that applied biotechnology offers, one of the most feasible is the evaluation and conservation of genetic resources (Toribio and Celestino, 2000).

Outstanding phenotypes are very interesting because they may represent specific combinations of genes that confer those trees features that can be of great interest. In addition, trees with rare genotypes that are in endangered populations should be conserved for the possible use of next generations. Therefore, the propagation of these trees conserving their specific genotypes would be of paramount importance. However, for most forest species vegetative propagation of adult valuable trees is presently not possible.

Plant regeneration using *in vitro* tissue culture techniques gives the possibility to clone desired individuals for conservation and clonal forestry purposes. In particular regeneration by somatic embryogenesis is been currently applied, even at the commercial level (Sutton, 2002). Also, methods to clone adult are by somatic embryogenesis, which presumably involve complete rejuvenation, are beginning to be reported (Celestino et al., 2005).

The cork oak is one of the most widely spread tree species in the Western Mediterranean region. Within this area, the Balearic Islands are considered to be a glacial refuge, and therefore a reservoir of genetic resources (López de Heredia et al. 2004). Cork oak populations in Balearics are very small and isolated, that likely imply high level of endogamy affecting their future stability. Therefore it is necessary to apply *in situ* and *ex situ* conservation strategies. A singular tree has been found in the small Minorca Island population. The haplotype of this Alfavaret's tree is of Tyrrhenian origin that makes this tree different to other cork oak trees found in that island, and suggests a past link between Minorca and Sardinia (López de Heredia et al. 2004). Moreover, this tree do not bear a deletion within an ITS from ribosomic nuclear DNA, which is fairly common in many populations of this species, and indicates that it may be the descendant of a very ancient population (López de Heredia, pers. com.). This tree is currently in a precarious condition, severely affected by fungal pathogens, and it has not produced acorns in the last years. Hence there is a clear need of vegetative propagation. We have developed methods to clone adult cork oak trees by somatic embryogenesis (Hernández et al., 2001), and hence the aim of the present work was to determine whether established methods could be used to clone this singular tree. Therefore, this study deals with one of the most relevant application of the *in vitro*

tissue culture techniques, the plant regeneration for *in situ* and *ex situ* conservation of forest genetic resources.

Material and Methods

Three branches were collected from the crown of the endangered cork oak (*Quercus suber* L.) tree growing in a natural forest close to Alfavaret (Minorca island, Spain) in November 2004, and sent to the laboratory wrapped in plastic. Pieces of branches of about 15 cm length and between 1 - 3 cm diameters were cut and immersed in a dissolution with 1 g/l of each Benoagrex® (50% benomyl) and CaptosanR® (8% carbendazim plus 40% captan) for 10 min. They were placed in a growth chamber at 25 ºC, high relative humidity (80-95%) and a 16 h photoperiod for sprouting. Expanding leaves were harvested and surface sterilised with 70% ethanol for 30 sec ensued by immersion in a 10% sodium hypochlorite solution (3.5% active chlorine) plus two drops of Tween 20 for 10 min, followed by three rinses with sterile distilled water. They were cultured following the improved protocol previously defined (Hernández et al., 2003a,b). Briefly, after culture in a preconditioning medium for 7 days, the induction protocol consisted of three phases, all of them on the same basal medium. This was based on the macronutrients from Schenk & Hildebrandt (1972) and the other components from Murashige & Skoog, (1962). The primary induction phase included 50 µM of BA and 10 µM NAA and was performed in darkness for 30 days. In the secondary induction phase the regulators were reduced to 0.5 µM of BA and 0.5 µM NAA, and was carried out in a 16 h photoperiod at 25 ±1 ºC for 30 days. The manifestation phase was accomplished on medium lacking growth regulators, under the preceding growing conditions. All the conditions of this last phase were also used for the proliferation of embryogenic lines by recurrent embryogenesis, as described elsewhere (Fernández-Guijarro *et al.*, 1995).

Leaves from the three branches were cultured separately, and therefore three embryogenic lines were distinguished. These lines were actively amplified for about 5 months by monthly subculture to fresh medium. From them, somatic embryos that matured spontaneously (white opaque, 15-20 mm length, average fresh weight 225 mg) were picked out and cold stored (4 ±1 ºC) in darkness for 60 days. Then, they were transferred to the same semi-solid basal medium without regulators for germination, following the protocol previously defined (Hernández el al., 2003b). After 30 days, germinating embryos were placed in forest containers with substrate (1 peat: 3 pine bark: 1 sand) for gradual weaning in a growth chamber with high relative humidity. Acclimatized somatic seedlings carry on developing in a greenhouse.

Three yield parameters (frequency of sprouting, frequency of leaves forming somatic embryos, and frequency of germination) were recorded, and the Chi-square test of independence was applied to detect significant differences among plant material coming from the three branches.

Results and discussion

Without any noticeable difference with the previous experience (Hernández et al., 2001; Hernández et al., 2003a,b) all the established protocol performed well with plant material from the Alfavaret's tree. Sprouting of epicormic shoots from pieces of branches was able to

provide enough expanding leaves for induction of somatic embryogenesis. First leaves could be collected from sprouted shoots 15 days after placing branches in the growth chamber. Pieces of branches sprouted at 26 % in average, ranging from 17 to 43 % depending on the branch, but these differences were not significant (Table 1). Epicormic shoots lasted for about one month giving a moderately abundant crop of new expanding leaves for induction purposes.

Branch	Number of pieces	Sprouting (%)	Number of leaves	Embryogenesis (%)
A	34	17.6	63	17.5
B	23	43.5	142	22.5
C	50	24	33	54.5
Total	107	26.2	238	25.6
χ^2 test (Df = 2)		χ^2 = 2.71; p = 0.258		χ^2=8.79; p = 0.012

Table 1. Sprouting of epicormic shoots in pieces of three different branches from the crown, and frequency of induction of somatic embryogenesis in leaves from the Alfavaret's cork oak tree.

First somatic embryos arose at the end of the culture period on medium with low concentration of plant growth regulators. However, the maximum response of somatic embryo formation was obtained after one month on medium lacking regulators. About 25 % of the uncontaminated leaves gave somatic embryos in average. There were significant differences in the behaviour of leaves from the different branches, their frequencies ranging from 17 to 54 % (Table 1). This is a novel factor influencing somatic embryogenesis not previously detected. Probably the different branch's condition within the individual tree affects the ability of leaves to form somatic embryos, as occurs when branches are taken from the same tree in different times in a year, and even in different yeas as previously reported (Hernández et al., 2003b and unpublished results). The frequency of induction of somatic embryogenesis of the Alfavaret's cork oak tree was similar to the average frequency obtained from selected cork oak trees growing in La Almoraima (Cádiz, Spain) when plant material was collected in spring (Hernández et al., 2003b).

Branch	Number of somatic embryos	Germination (%)	Number of somatic seedlings
A	11	45.5	5
B	31	45.2	14
C	10	50	5
Total	52	46.2	24

Table 2. Germination of somatic embryos cloned from the Alfavaret's cork oak tree.

Embryogenic lines from the Alfavaret's tree were easily multiplied by secondary embryogenesis in a recurrent process without apparent decline, as happened previously with

embryogenic lines of this species (Fernández-Gujarro et al., 1995). They showed the same pattern of development, without any qualitative difference.

Germination performed equally. About 46 % of the spontaneously matured somatic embryos germinated in average, without differences among the three embryogenic lines (Table 2). Cork oak somatic embryos that did not undergo a specific maturation treatment germinated in percentages ranging from 26 to 55 % (Hernández et al., 2001; Hernández et al., 2003b), and therefore the tree considered in this study falls within this range.

A low number of somatic seedlings were produced (Table 2) that will be used for conserving dynamically this valuable genotype, both *in situ* and *ex situ*.

Acknowledgements

We are indebted to the *Servicio de Gestión Forestal* (*Gobierno Balear*) for providing plant material and to Unai López de Heredia for giving genetic information. Technical assistance of Nuria Cleto, Noelia Ramírez and Sara Espinosa is highly appreciated. Financial support was supplied by the national project RTA02-032, and by the private company "Grupo Empresarial ENCE".

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